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TITLE:

DETECTION OF ESTROGEN RECEPTOR-β AND METHODS FOR DIAGNOSIS AND CLASSIFICATION OF CANCER

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DETECTION OF ESTROGEN RECEPTOR-β AND METHODS FOR DIAGNOSIS AND CLASSIFICATION OF CANCER

Background of the Invention

The effects of estrogens in biological systems are varied. The effects of these hormones are mediated by a family of receptors known as the estrogen receptors that include estrogen receptor-α (ERA) and estrogen receptor-β (ERB). The estrogen receptor was first cloned in 1986 and at that time only one estrogen receptor had been identified, now known as ERA. Recently, the second receptor isoform, ERB, was discovered in rat prostate and ovary tissue (Kuiper, GGJM et al. 1996. *Proc. Natl. Acad. Sci. USA* 93:5925-5930). This second isoform estrogen receptor has now been cloned in human and mouse, with the human gene for ERB mapped to chromosome 14q22-24 (Enmark, E. et al. 1997. *J. Clin. Endocrinol. Metab.* 82:4258-4265). The ERB protein is a 54.2 kDa protein in humans (Chang, W.Y. and G.S. Prins. 1999. *The Prostate* 40:115-124).

ERA is a useful clinical biomarker of breast cancer progression and is routinely monitored by clinicians in order to estimate patient prognosis and select optimal therapies. In guidelines published by the ASCO Tumor Marker Expert Panel (Ravdin, P.M. 1997. *Prognostic factors in Breast Cancer*, American Society of Clinical Oncology Educational Book, p. 217-227), the estrogen receptor (i.e., ERA) and the progesterone receptor were the only biomarkers recommended for routine use in management of patients with breast cancer. The estrogen receptor assay is most useful as a tool to

aid in selection of therapies for breast cancer. Patients having an estrogen-receptor negative tumor seldom respond to endocrine therapy.

Predicting the probability of response to endocrine therapy in estrogen-receptor-positive patients is more difficult. Overall, approximately 50-60% of women with estrogen-receptor-positive breast cancer will receive some degree of benefit from endocrine treatment with the anti-estrogen tamoxifen (1992. *Lancet* 339:1-15). Unfortunately, many patients who initially respond to tamoxifen will eventually recur with estrogen hormone-resistant disease.

Both ERA and ERB are part of a superfamily of nuclear hormone receptors that function as transcription factors when they are bound to their respective ligands. Nuclear receptors share common structural and functional features. ERA contains 595 amino acids with a central DNA binding domain (DBD) along with a carboxy-terminal hormone binding domain (HBD). ERB is somewhat shorter than ERA and is predicted to contain 530 amino acids (Ogawa, S. et al. 1998. *Biochem. Biophys. Res. Commun.* 243:122-126). The region of highest homology between ERA and ERB is in the DBD (97%), with much less homology in the HBD (54%). There is even less conservation in the amino-terminal activation function-1 domain (AF-1). Since estrogen-receptor-mediated gene transcription is stimulated through the two transactivation domains, AF-1 and the carboxy-terminal AF-2 domain (Kumar, V. et al. 1986. *EMBO J.* 5:2231-2236; Kumar, V. et al. 1987. *Cell* 51:941-951; Tora, L. et al. 1989. *Cell* 59:477-487), ERB may regulate different genes from ERA and molecular mechanisms regulating ERB activity are likely distinct from those regulating ERA activity.

In fact, studies have shown that ERB has negligible autonomous AF-1 activity as compared to ERA when estrogen response element-based reporter activity is examined (Cowley, S.M. and M.G. Parker 1999. *J. Steroid Biochem. Mol. Biol.* 69:165-175; Hall, J.M. and D.P. McDonnell. 1999. *Endocrinology* 140:5566-5578), a result that explains the lack of agonist activity exhibited by tamoxifen with ERB on artificial estrogen response element-based reporter systems. Further, the three dimensional structure of the ERB HBD indicates that its AF-2 co-activator contact surface is similar but not identical to ERA (Pike, A.C. et al. 1999. *EMBO J.* 18:4608-4618). These data suggest that there is a differential recruitment of cellular factors, such as estrogen receptor co-regulatory proteins, to the transcription complex or preferential binding of some co-regulatory proteins to ERB when ERB is expressed.

The differences in ERA and ERB in the ligand binding domain have led to examination of the ligand-binding profiles of ERB versus ERA. Studies have shown that these two receptors share similar profiles in terms of specificity and affinity for compounds such as estrial, estrone, estradiol-17β, as well as many other known estrogen receptor ligands. However, significant differences in ERB and ERA binding and activation have been identified. The selective estrogen receptor modulator raloxifene binds ERB with an affinity four times lower than ERA (Kuiper, GGJM et al. 1998. *Endocrinology* 13:4252-4263). Regulation of ERB-mediated gene transcription at AP1 enhancer elements has been shown to be different than regulation of ERA (Paech, K. et al. 1997. *Science* 277:1508-1510). Also, unlike ERA, ERB transcriptional activity is inactive with estradiol (Paech, K. et al. 1997. *Science* 277:1508-1510). These differences in ERB binding and transcriptional activity may be related to selective activity of the receptor in different tissues. ERB

has been identified in prostate tumor tissue, testis, ovary, thymus, spleen, breast, adipose tissue, uterus, pituitary adenomas, and kidney.

The physiological function of ERB has not been fully elucidated. Using ERB knockout mice, it has been shown that ERB is important for growth control of the epithelium of the urogenital tract and that ERB expression may afford protection against hyperproliferation and carcinogenesis (Gustafsson, J. 1999. *J. Endocrinol.* 163:379-383). These results in animals are supported by clinical findings (Leygue, E. et al. 1998. *Cancer Res.* 58:3197-3201; Pujol, P. et al. 1998. *Cancer Res.* 58:5367-5373).

In some circumstances and under certain conditions, ERB interferes with the estrogen responses elicited by stimulation of ERA (Hall, J.M. and D.P. McDonald. 1999. *Endocrinology* 140:5566-5578). It has been found that ERB expression can inhibit tamoxifen's agonistic effects on ERA in breast cancer cells. Further, a study of 40 tumor samples showed that ERB expression was significantly lower in progesterone receptor-positive tumors, indicating a role for ERB expression in tumor prognosis determinations since progesterone receptor is a biomarker and predictor of response to tamoxifen therapy (Dotzlaw, H. et al. 1999. *Cancer Res.* 59:529-532).

What is needed are methods and materials to independently assess both the ERA and ERB status of breast tumor cells in order to define more specifically the clinical outcome of a patient. ERB expression may correlate with a poor prognosis for response to tamoxifen therapy and would provide a valuable tool in determining the appropriate therapy for breast cancer patients. In fact, the difference in expression of ERA versus ERB has been suggested to be important in the progression of breast cancer (Fuqua, S.A.W. et al. 1999. *Cancer Res.* 59:5425-5428).

Antibodies, both monoclonal and polyclonal, have been developed against the estrogen receptor that are not selective for ERA versus ERB. Traish (US Patent 5,744,356) describes a monoclonal antibody that is specific for an epitope within residues 1-184 of an estrogen receptor protein that is useful for detection of the activated 4S and 5S forms of the estrogen receptor, distinguishing between the hormone-activated form of the receptor and the native or unactivated form of the receptor. In addition, monoclonal and polyclonal antibodies against ERA are available commercially (Novocastra Laboratories Ltd., Newcastle upon Tyne, England). Antibodies developed to date have not solved the need for independent methods and materials allowing simultaneous detection of the two receptors in a tissue.

Polyclonal antibodies against ERB are commercially available (Alpha Diagnostic International, Inc., San Antonio, TX). These commercially available polyclonal antibodies are directed to the carboxy terminus of the ERB receptor protein.

Fuqua et al. (1999. *Cancer Res.* 59:5425-5428) reported development of ERB-specific antibodies for characterization of ERB protein expression in breast cancer cell lines and tumors using Western Blot analysis. In this report, monoclonal antibodies were made to a peptide representing the first 18 amino acids (N-terminal region) of the longest ERB open reading frames reported (amino acids 39-146 of the ERB protein) while polyclonal antibodies were made against a peptide within the ERB B domain. To date, all of the ERB antibodies reported can only be used to detect the presence of ERB in frozen tissue samples; none of the antibodies, either polyclonal or monoclonal, bind to paraffin-embedded, fixed tissue samples.

Since most pathological samples are fixed and embedded in paraffin for storage, there is a need for methods of ERB detection that are specific for the beta form of the estrogen receptor and that can be used in immunocytochemical studies of paraffin-embedded fixed tissue sections. In order to better understand the role of ERB in tissues, methods and materials for detection that are specific for this receptor over ERA are needed that are useful in fixed histological specimens.

Background For Extension of Usefulness of Anti-ER-Beta Antibodies

Nuclear Receptors.

Nuclear receptors are ligand activated transcription factors that have been shown to play critical roles in development, cell growth, cell differentiation and homeostasis (Mangelsdorf, D.F. et al. 1995. *Cell* 83:835-839). The nuclear receptor superfamily can be subdivided into steroid receptors (estrogen, progesterone, etc.,) and nonsteroid receptors thyroid, retinoic acid, etc.) (Beato, M. et al. 1995. *Cell* 83:851-857; Mangelsdorf, D. et al. 1995. *Cell* 83: 841-850). All nuclear receptors have similar, but not identical, structure which can be divided into several functional domains (Mangelsdorf, D. et al. 1995. *Cell* 83: 841-850). This includes a DNA binding domain which is highly conserved among all the nuclear receptors and two transcriptional activation domains. The transcriptional activation domain near the N-terminal is constitutively active and is known as AF-1 (activation function 1). The second transcriptional activation domain, located in the C-terminal region, is much stronger and hormonally regulated. It is known as the AF-2 (activation function 2). The AF-2 domain has unique conformations as an aporeceptor, agonist bound receptor or antagonist

bound receptor. Unliganded steroid receptors are complexed with chaperone proteins such as the heat shock proteins and remain inactive (Smith, D.F. et al. 1992. *J Biol Chem* 267:1350-1356). Upon binding of ligand, the receptor forms homodimers and binds to DNA. In contrast, nonsteroid receptors are unliganded heterodimers, already bound to DNA and are complexed with corepressor complexes which inhibit gene transcription (Magelsdorf, D. et al. 1992. *J Biol Chem* 267:1350-1356). Upon the addition of ligand, the corepressor nuclear receptor complex is destabilized and transcription is activated. These changes in conformation when agonists bind allow other proteins, coactivators, to interact with the receptor to produce an activated transcription factor. The exact composition of these nuclear receptor coactivator/corepressor complexes in the cell is not known, but a large number of proteins have been shown to interact with nuclear receptors on the basis of direct biochemical interaction both *in vivo* and *in vitro* and have been termed coactivators, corepressors and integrators (Darimont, B.D. et al. 1998. *Genes & Dev* 12:3343-3356; Wong, C. et al. 1998. *Mo & Cell Biol* 18:5724-5733; Horwitz, K.B. et al. 1996. *Mol Endo* 10:1167-1177). These factors add another level of complexity in the mechanism of transactivation of nuclear hormone receptors with the amounts and combinations of these cofactors being extremely important in the regulation of the receptor activity.

Estrogen Receptor Hetero Dimers and Isoforms

The ER-α and ER-β proteins have been shown to form homodimers and bind DNA with equal affinity (Ogawa, S. et al. 1998. *Biochem. Biophys. Res. Comm.* 243:122-126; Petterson, K. et al. 1997. *Mol. Endo.* 11:1486-1496). In addition experiments have shown that ER-α and ER-β

heterodimers can form both *in vitro* and in intact cells. This suggests that two new estrogen signaling pathways exist in cells that exclusively express ER-β via homodimer formation in cells that exclusively express one receptor subtype and homo- and hetero-dimer formation in cells that express both receptor subtypes. With the findings that levels of both estrogen receptors and isoforms are altered in tumors leads to the possibility that the change in protein levels alter the potential ER dimers that may form and their activity (Leygue, E. et al. 1999. *Cancer Research* 59:1175-1179). Indeed, ER-βcx is truncated at the C-terminal end, but contains an extra 26 amino acids due to an alternative splicing and it no longer binds ligand. But, ER-βcx can form heterodimers with ER-α and therefore inhibit DNA binding and ER-α mediated estrogen transcription by acting as a dominant negative receptor (Ogawa, S. et al. 1998. *Nuc. Acids Res.* 26:3505-3512).

Similar to other nuclear receptors both estrogen receptors have been shown to have additional isoforms generated by alternative splicing (Murphy, L.C. et al. 1997. *Ann. Medicine* 29:221-224; Shupnick, M.A. et al. 1998. *Mol. Cell. Endo.* 138:199-203; Moore, J.T. et al. 1998. *Biochem BioPhys. Res. Comm.* 247:75-75). These alternative splice variants again add an additional level of complexity to understanding estrogen action and the biological properties of a tumor's response to estrogen and antiestrogen treatment (Shupnick, M.A. et al. 1998. *Mol. Cell. Endo.* 138:199-203). This includes five isoforms that have been reported at the mRNA level for ER-β (Figure 4)(Moore, J.T. et al. 1998. *Biochem BioPhys. Res. Comm.* 247:75-75). The figure indicates the carboxyl terminal end of the ER-β protein and indicates alterations in primary sequences due to alternative splicing of the mRNA. This variation in amino acid sequence will be used to generate synthetic

peptides for antibody production.

Estrogen Receptors in Cancer.

The cloning of the ER-β receptor has prompted investigators to reevaluate the molecular basis for estrogen action and the role of estrogen receptors in tumors from tissues where ER-β is normally expressed and in breast cancer (Speirs, V. et al. *Cancer Res.* 59:525-528). Most studies to date have had to use RT-PCR to measure mRNA levels rather than immunohistochemistry (IHC) to determine ER-β protein levels because of lack of ER-β specific antibodies that work in IHC (Hanstein, B. et al. 1999. *Mol. Endo.* 13:129-137). Therefore, all these results are tempered by the fact that it is not known if there is a direct correlation between the levels of mRNA and the level of protein for ER-β in normal and/or tumor tissue. Full length ER-β has been shown to be expressed in normal breast tissue and human breast tumors (Dotzlaw, H. et al. 1997. *Endocrin Meta.* 82:2371-2374). The ratio of ER-α and ER-β change during tumor grade suggesting that both receptor types have a role in tumor formation (Leygue, E. et al. 1998. *Cancer Res.* 58:3197-3201). Recently a PCR assay was developed using triple primer sets for the co-amplification of ER-β and several isoforms. The results indicate that at the mRNA level changes in the relative expression of the full length and several isoforms occurs during breast tumorigenesis and cancer progression (Leygue, E. et al. 1999. *Cancer Research* 59:1175-1179; Leygue, E. et al. 1998. *Cancer Res.* 58:3197-3201).

Very recently two studies have been done to compliment the RNA studies previously reported. In the first study by Fuqua et al., they have shown at the protein level that ER-β and several

isoforms are expressed in breast cancer cell lines and tumors and correlated this to RNA levels (Fuqua, S.A.W. et al. 1999. *Cancer Res.* 59:4525-4528). In the second study by Jarvinen, et al. (Jarvinen, T.A.H. et al. 2000. *Am J Pathology* 156:29-35) they used a polyclonal antibody (PA1313, Affinity Bioreagents, Golden, CO) on frozen sections to analyze ER-β expression by immunohistochemistry (IHC). Their results indicate that ER-β is expressed in normal epithelial cells and in 60% of breast tumors and ER-β is often coexpressed with ER-α and PR. They conclude that ER-β can be used along with ER-α and PR to determine if a tumor will respond to hormone therapy. It is important to note in this study that the tumor sections were frozen and their attempts to use paraffin-embedded tissue (the way most clinical samples are stored) did not work (Jarvinen, T.A.H. et al. 2000. *Am J Pathology* 156:29-35).

Estrogen Receptor in Hormone Therapy

The mRNA level of ER-β accessed by PCR in tumors showed that ER-β levels were much lower in progesterone receptor positive (PR+) tumors as compared to PR- tumors and ER-β levels can be reduced by progestin in breast cancer cells (Dotzlaw, H. et al. 1999. *Cancer Res.* 49:529-532). In comparing ligand binding of ER-α and ER-β, both bind physiological ligands and some antagonists with equal affinity, but differ in there ability to bind and affect gene transcription in response to other specific antiestrogens SERMs and environmental estrogens (Tremblay, A. et al. 1998. *Endo* 139:111-118; Giguere, V. et al. 1998. *Steroids* 63:335-339, 1998). ER-β binds to estrogen metabolites with similar affinity as ER-α, including estrogen, diethylstilbesterol, estriol and estrone. Binding of estrogen antagonists such as raloxifene and tamoxifin were also identical

(Kuiper, G.G.J.M.m et al. 1997. *Endocrinology* 138:863-870). A difference in binding affinity does occur in that ER-β binds xenoestrogens and phytoestrogens (genistein) significantly better than ER-α (Kuiper, G.G.J.M. et al. 1998. *Endocrinology* 13:4252-4263) indicating that ER-β plays a unique role in the physiologic functions of natural estrogens.

Selective estrogen receptor modulators (SERMs) have been shown to act as either ER antagonists or agonist depending on the type of tissue analyzed and the dose administered. An example is the new SERM raloxifene which acts as an antiestrogen in breast tumor tissue and brain, but it maintains estrogen-like effects in bone and heart and has actually shown an increase in bone density, but does not stimulate endometrial growth or increase the chance of breast cancer (Raafat, A.M. et al. 1998. *Endo.* 140:2570-2580). This is similar to tamoxifen except that tamoxifen is an antagonist in brain tissue and also exerts estrogenic activity in endometrium to increase the cancer risk. The complexity of the problem is also effected in tissues where both receptors are expressed with the varying ratios of ER-α and ER-β and their isoforms may result in changing ratios of ER-α and ER-β homo- and hetero- dimers. These experiments along with the studies described above raised the possibility to the present inventors that ER-β is potentially a useful marker in determining hormone therapy responsiveness in some breast tumors. This inverse relationship between PR, which is a good prognostic variable and marker of response to hormone therapy may suggest that ER-β levels or increases in its expression may correlate with a poorer prognosis (Dotzlaw, H. et al. 1999. *Cancer Res* 59:529-532). Again these studies results need to be confirmed at the protein level.

Prognostic and Predictive Indicators of Cancer.

Morality from breast cancer has declined this decade due to earlier diagnosis and the widespread use of adjuvant chemotherapy and hormonal therapy after surgery. About 60% of patients with advanced breast cancer respond to chemotherapy with partial tumor regression, but only 15-20% have complete tumor regression. In early breast cancer, about 50% of those patients estimated to have a poor prognosis are now treated with several months of adjuvant chemotherapy. Recurrence and mortality are reduced only by 20-40% in such patients. Thus, many patients treated with toxic drugs do not stand to benefit because of tumor drug resistance, while others are not offered chemotherapy because they are mistakenly thought to have a good prognosis. Identifying the unknown changes or specific genetic alterations in an individual patient's tumor that correlate with tumor aggressiveness and/or response to treatment would have profound clinical implications.

Prognostic markers are those molecular features that correlate with the natural aggressiveness of a tumor, and predictive markers are those that correlate with treatment response. The estrogen receptor protein is a good example of both: ER+ tumors are more indolent, and they are also much more responsive to hormonal treatments such as tamoxifen. Overexpression of the HER-2/*neu* oncogene correlates with more rapid tumor recurrence, but also with a better response to doxyrubicin-based chemotherapy (Muss, H.B. et al. 1994. *N. Engl. J. Med.* 330:1260-1266; Seshadri, R. et al. 1993. *Clin. Oncol.*, 11:1936-1942; Slamon, D.J. et al. 1989. *Science* 244:707-12).

Breast cancer is a very common disease that afflicts millions of women worldwide (Slamon, D.J. et al. 1989. *Science* 244:707-12; Eisinger, F. et al. 1998. *Annals of Oncology* 9:939-950). Unfortunately, the clinical course of primary breast cancer varies considerably from patient to patient. Some patients have very long disease-free survival, while others experience a rapid deterioration with early recurrence of their breast cancer, followed shortly by death. Due to the complexity and heterogeneity of these tumors, determining the proper treatment presents several dilemmas for the physician and patient. Among these is the question of whether or not to use systemic adjuvant therapy following surgery. And, if so which therapy – hormonal, chemotherapy, or both – is best for a particular patient? In order to choose the best treatment for a specific patient, a physician must firsts determine the likelihood that the patient will have a recurrence of disease if no systemic therapy is administered after surgery.

Prognostic data is the key to this decision. Some of the disease variability is undoubtedly explained by differences in tumor growth rates, invasiveness, metastatic potential and other mechanisms that we do not fully understand. It is imperative that we develop biomarkers that measure these characteristics, either directly or indirectly, so those individual patients can be classified into subsets with varying risk of disease recurrence.

Three clinical situations for which prognostic factors would be useful have been described previously (Elledge, R.M. et al. 1994. *Cancer Research* 54:3752-3757). The first involves identifying patients whose prognosis is so good following surgery alone that the benefits of systemic

adjuvant therapy would not out-weigh the toxic effects. Approximately 70% of node-negative patients and 25% of node-positive patients remain alive for at least 10 years after surgery without any other therapy (Hilsenbeck, S.G. et al. 1992. *Human Pathology* 23:601-602; Hilsenbeck, S.G. et al. 1992. *Breast Cancer Res. Treat.* 22:197-206). The issue is whether subsets of patients with extremely low risk of disease recurrence can be identified so that chemotherapy can be avoided.

The second situation involves identifying patients whose prognosis is so poor even with conventional chemotherapy treatment that more aggressive forms of therapy might be warranted. In spite of improvements in adjuvant therapies, there are some patients whose cancers rapidly recur following conventional adjuvant therapy. These patients might be candidates for more aggressive regimens such as high-does chemotherapy followed by bone marrow or peripheral stem cell rescue. Our collaborators have already begun to address this problem by creating models to identify such patients.

The third, and perhaps most important, is the use of prognostic markers to predict which patients are or are not likely to benefit from specific therapies. While it is useful to accurately predict the likely clinical outcome of a patient's breast cancer, it is even more important to be able to alter the natural history of disease and to prevent or delay its recurrence. Therefore, biomarkers that are predictive of the utility of specific therapies, including specific chemotherapeutic agents, would be extremely useful in the clinic.

Thus, it is critical that monoclonal antibodies, and the hybridomas that produce them, that specifically bind estrogen receptor-β protein by located and characterized. In particular, such antibodies should specifically bind to an epitope within amino acid residues 1-146 or to an epitope within amino acid residues 1-36 of the estrogen receptor-β protein, and even more particularly that such antibodies effectively bind to the receptors in fixed paraffin-embedded tissue samples. With such reagents, it would be desirable to build kits for the detection of receptor-β protein in a biological sample. It would also be highly advantageous to utilize ER-β as a marker in determining hormone therapy responsiveness in breast tumors. It would also be a major advancement if reagents, tests and kits were feasible wherein, depending on the level of expression, each ER subtype could be evaluated for its impact on the ability of a specific tumor type to respond to clinically important anticancer agents. It might be possible, using such invention to determine the protein distribution and function of ER-β and its isoforms in various estrogen responsive tissues. If so, such inventions would be critical to predicting pharmacologic and physiologic impact as well as to facilitate the development of tissue specific estrogen agonists and antagonists to treat the disease in only the affected tissue. Additionally, using such inventions, it might be possible to understand the mechanisms of action of both ER types and to help develop superior SERMS than those currently available (tamoxifen, raloxifene). It would be especially valuable to be able to block estrogen action in a specific tissue where it promotes growth and possibly cancer (breast) but does not effect estrogen action on bone and brain where it protects women from osteoporosis and Alzheimers disease, respectively. These inventions will be crucial in addressing the challenge to eliminate the

adverse effects of current selective estrogen receptor modulators (SERMs) and to minimize the problems with hormone replacement therapy.

Summary of the Invention

The present invention is directed to a monoclonal antibody that specifically binds estrogen receptor-β protein.

One aspect of the invention is a monoclonal antibody that specifically binds to an epitope within amino acid residues 1-146 of the estrogen receptor-β protein in fixed paraffin-embedded tissue samples.

Another aspect of the invention is a monoclonal antibody that specifically binds to an epitope within amino acid residues 1-36 of the protein. A preferred embodiment of the monoclonal antibody being an anti-estrogen receptor-β produced by hybridoma clone 14C8.

Yet another aspect of the invention is a hybridoma that secretes a monoclonal antibody antibody that specifically binds to an epitope within amino acid residues 1-146 of the estrogen receptor-β protein in fixed paraffin-embedded tissue samples.

Still yet another aspect of the invention is a kit for the detection of receptor-β protein in a biological sample. Still another aspect of the invention is the use of ER-β as a useful marker in determining hormone therapy responsiveness in breast tumors.

Still another aspect of the present invention is the determination of the level of expression for each ER subtype and the impact thereon on the ability of a specific tumor type to respond to clinically important anticancer agents. Use of the reagents, tests and kits of the present invention to determine the protein distribution and function of ER-β and its isoforms in various estrogen

responsive tissues is yet another aspect of the invention that allows predicting pharmacologic and physiologic impact of a therapy, as well as to facilitate the development of tissue specific estrogen agonists and antagonists to treat the disease in only the affected tissue.

Description of the Drawings

Figure 1 depicts a schematic protein map of the homologous regions of ERA and ERB and indicates the regions of ERB targeted for monoclonal antibody development.

Figure 2 illustrates the binding of monoclonal antibodies directed against ERA and ERB to recombinant ERB.

Figure 3 illustrates the binding of monoclonal antibodies directed against ERA and ERB to recombinant ERA.

Figure 4 illustrates the comparison of the deduced carboxyl terminal amino acid sequences of ER-β and specific isoforms that have been isolated from tumor cell lines and tissue.

Figure 5 illustrates immunocytochemical analysis of paraffin-embedded, fixed tissue processed with 14C8 ER-β monoclonal antibody. A. Endocervical epithelium. B. Inflitrating lobular carcinoma of the breast.

Detailed Description of the Invention

ERB-specific monoclonal antibodies have been developed that, unlike previous antibodies against ERB, have both high affinity and specificity for ERB and can be used to detect the presence of ERB in paraffin-embedded fixed (permanent) tissue sections. Such permanent tissue section

samples are the type of tissue sections that are archived. Therefore, the present invention provides a valuable clinical tool for examining the role of ERB in tumor progression in archival sections from prospective clinical studies, where "archival" tissue sections are defined as sections that have been fixed and/or paraffin-embedded.

Specific antibodies to ERA are commercially available. Such ERA antibodies, when used with the ERB specific antibodies of the present invention provide a basis for a method of detection of ERB in tissues that express both ERA and ERB and allow for quantification of the levels of expression of ERB as compared to ERA. Studies have suggested that ERB expression indicates a poor prognosis to hormone therapy for certain cancers (the opposite of ERA expression). Therefore, the identification of the expression ratio of ERB/ERA in tissues is useful in the diagnosis and classification of diseases associated with estrogen receptor expression, such as breast cancer.

With the recent identification of several variants of ERB in human tissues, five at this time (Moore, J.T. et al. 1998. *Biochem. Biophys. Res. Commun.* 247:75-78; Leygue, E. et al. 1999. *Cancer Res.* 59:1175-1179), it has become clear that choosing the correct target region for monoclonal antibody development is critical. This is because the various isoforms are formed by shortening of the carboxy terminal region of the ERB protein. Therefore, antibodies directed to the carboxy terminal region probably recognize only certain isoforms of the ERB protein, rather than the total levels of ERB. In addition, as illustrated in Figure 1, the amino acid sequence of the carboxy ends of ERA and ERB are greater than 95% homologous, while the amino acid sequence of the amino ends of ERA and ERB are much less homologous.

The inventors reasoned that an antibody that is directed to the N-terminal region of the ERB protein may be more specific for ERB and would recognize all known ERB variants to provide a more accurate assessment of total ERB levels. But, it was not known if antibodies to this region would be successful in histological samples. In the present invention, the N-terminal region of the ERB protein was targeted for monoclonal antibody development.

The resultant antibodies provide an assessment of total ERB binding in a tissue section or biological sample. Several monoclonal antibodies, specific for the amino end of ERB, have been developed and the clones are known as 14C8, 4D2, 6A12, 6B12, and 14G2. These antibodies are directed to the first 146 amino acids of the ERB protein (Figure 1). For the monoclonal antibodies 14C8 and 4D2, the epitopes are located within the first 36 amino acids. For clones 6A12, 6B12 and 14G2, the epitopes are located within amino acids 36-146. The production of these monoclonal antibodies is set forth in more detail in Example 1.

The various monoclonal antibodies developed have been tested for their affinity and specificity for binding ERB in both ELISAs (see Example 2), western blots (see Example 3) and immunocytochemical techniques (see Example 4). These methods are standard screening methods used to characterize the specificity and affinity of antibody binding.

The present invention includes both compositions and methods for detecting ERB in biological samples. In the context of the invention, "biological samples" would include but not be limited to tissue samples taken from a patient for the purpose of pathology examination. These samples could be tumor tissue samples as well as non-tumor tissue samples, and can be fresh samples as well as archival samples. Other types of biological samples could be tested for the

presence of ERB using the compositions and methods of the present invention and would include fixed cell cultures or cell preparations, as well as gels run from samples prepared from tissue or cell samples.

The monoclonal antibodies of the present invention can be used for the detection of ERB in biological samples using known methods for detection of antibody binding. Such methods, include but are not be limited to, radioimmunoassays, immunoprecipitation, western blotting, enzyme-linked immunosorbent assays (ELISA), and immunohistochemistry or immunocytochemistry. Example 5 describes the use of these monoclonals in immunocytochemical kits for the analysis of tissue samples.

The following non-limiting examples are provided to better illustrate the claimed invention.

EXAMPLES

Example 1: Monoclonal Antibody Preparation

The antigen used for production of monoclonal antibodies was expressed from a cDNA fragment from the 5'-end of ERB and was made from total RNA extracted from the human breast cancer cell line MCF-7. The 5' PCR primer was GTXERβ-F1: 5'-ctggatcc ATG GAT ATA AAA AAC TCA CCA TC –3' (NT#410-441 from GenBank Sequence AF51427) and the 3' PCR primer was ERY-5(RC) Sal I 5'gcgtcgac TGA GCA TCC CTC TTT GAA CCT GGA C –3'(NT# 833-864 from GenBank Sequence AF51427) (upper case letters denote the protein coding sequence while lower case letters denote the untranslated sequence). For cloning and expression of the antigen, the PCR ERB cDNA fragment was digested with appropriate restriction enzymes and then purified from

gels. The purified DNA fragment was cloned into pET28a+ expression vector (Novagen, Madison, WI). After screening and characterization of the initial clones, the positive clones were grown to a larger volume and the expressed protein was purified through His-Bind (Pierce, Madison, WI) column chromatography under denatured conditions to near homogeneity.

Five female Balb/c mice were immunized with purified recombinant ERB. The primary immunization antigen consisted of 50 μg of purified ERB protein suspended in 150 μl of phosphate-buffered saline (PBS) mixed with an equivalent concentration of Freund's complete adjuvant administered intradermally and intraperitoneally into mice. Booster immunizations contained 50 μg ERB protein and incomplete adjuvant. Sera from immunized animals was tested for the presence of immunoreactive antibody by ELISA and western blots against the purified recombinant ERB protein.

Spleens from mice producing immunoreactive antibody were fused to NS-1 myeloma cells using standard techniques. After fusion, cells were grown in HAT selective media. Supernatants from growing hybridoma were tested for ERB-specific antibodies by ELISA and western blotting using purified recombinant ERB protein as antigen. All cultures were examined for antibody secretion during the expansion of the clones.

Clones determined to be positive by ELISA and western blots were single-cell cloned. Hybridoma cells were grown from frozen stocks and were single-cell cloned by serial dilution methods. Clones grown from single cells were screened again by ELISA in order to prevent the possibility of multiple species of monoclonal antibody in the parental hybridoma culture. Cell lines were expanded and a portion was frozen to preserve antibody-producing cell lines. Once a larger volume of overgrown supernatant was obtained, the specific monoclonal antibodies were purified

and concentrated to obtain high-purity antibody stock.

Example 2: ELISA Analysis

The purified monoclonals were tested for their specificity and affinity. For example, Figure 2 illustrates the binding of two lots of 14C8 to ERB and Figure 3 illustrates the binding of the two lots of 14C8 to ERA. The analyses were done by a standard ELISA in 96 well microtiter plates. Each well contained 500 ng of purified ERB or ERA recombinant protein.

Purified anti-ERB 14C8 monoclonal antibody (lots 904 and 002), anti-ERA 1F3 antibody (lot 904), were normalized to a concentration of 1 mg/ml. Various dilutions of these antibodies, ERA polyclonal antibody (lot 904) and normal mouse serum (i.e., 1/250, 1/500, 1/1000, 1/2000 and 1/4000) were made and tested for their binding to recombinant ERA and ERB. Table 1 gives the binding data for these antibodies to both recombinant ERA and ERB in A_{405} which is proportional to the amount of antibody that bound to the 500 ng of protein in the well.

TABLE 1

Erβ antigen

Dilution	Erβ 14C8 (lot 904)			Erβ 14C8 (lot 002)			Era 1F3 (lot 904)			Era PA b (lot 904)			Normal Serum		
1/250	0.773	0.726	0.7495	0.728	0.726	0.727	0	0	0	0.129	0.065	0.097	0	0	0
1/500	0.513	0.469	0.491	0.571	0.485	0.528	0	0	0	0.028	0.005	0.0165	0	0	0
1/1000	0.303	0.248	0.2795	0.339	0.302	0.3205	0	0	0	0	0	0	0	0	0
1/2000	.0129	0.086	0.1075	0.143	0.117	0.13	0	0	0	0	0	0	0	0	0
1/4000	0.012	0.022	0.017	0.019	0.004	0.0115	0	0	0	0	0	0	0	0	0

Erα antigen

Dilution	Erβ 14C8 (lot 904)			Erβ 14C8 (lot 002)			Era 1F3 (lot 904)			Era PA b (lot 904)			Normal Serum		
1/250	0.017	0.0155	0.016	0	0	0	0.739	0.858	0.7985	2.881	2.73	0.097	0.047	0.055	0.051
1/500	0	0.004	0.002	0.003	0.007	0.005	0.539	0.394	0.4665	1.962	0.005	0.0165	0.022	0.023	0.0225
1/1000	0.009	0.248	0.0045	0.006	0.008	0.007	0.287	0.279	0.283	1.443	1.568	1.5055	0.009	0.025	0.017
1/2000	0	0.012	0.006	0	0.009	0.0045	0.278	0.12	0.199	0.97	0.888	0.929	0	0	0
1/4000	0.012	0.005	0.085	0.016	0	0.008	0.061	0.081	0.071	0.617	0.505	0.581	0	0	0

Example 3: Western Blot Analysis

The specificity of clones 14C8, 6B12, and 6A12 were analyzed by western blot assay techniques. Supernatants from each of the clones bound specifically to ERB and showed no cross-reactivity with ERA protein.

Cell or tumor lysates were used for the western blots. Cell pellets or tumor samples were homogenized in a high salt buffer (20 mM Tris-HCl, pH 7.5; 2 mM DTT, 0.4 M KCl; 20% glycerol) containing a mixture of protease inhibitors (2.5 μg/ml aprotinin, antipain, leupeptin, and pepstatin plus 0.3 mM phenylmethylsulfonyl fluoride; Sigma Chemical Co., St. Louis, MO.). Homogenates were then centrifuged at 100,000 x g for 1 hour, and the supernatants stored at -20° C until they were assayed. Homogenates (50-100 μg) were electrophoresed on 10% SDS-PAGE gels and transferred onto nylon membranes (Schleicher and Schuell, Keene, NH).

The blots were first stained with StainAll dye (Alpha Diagnostic International, Inc. San Antonio, TX) to confirm uniform transfer of all samples and then incubated in blocking solution (5% nonfat dry milk in TBST). After brief washes with TBST, the filters were then reacted with ERB monoclonal antibody at a dilution of 1:20 or 1:200 for 1 hour at room temperature, followed by extensive washes with TBST. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Corporation, Burlington, MA) for 1 hour, washed with TBST, and developed using an enhanced chemiluminescence process (Amersham Corporation, Burlington, MA). In order to determine levels of non-specific staining, primary antibody (i.e., anti-ERB antibody) was eliminated in some experiments and secondary antibody was incubated with the

samples by itself. Positive controls were run and included the use of the mammalian expression vectors (pSG5) containing the complete open reading frames of ERB1 and ERB2, which were translated *in vitro* in a 50 µl rabbit reticulocyte translation reaction (Promega, Madison, WI). Supernatants (5 µl) were analyzed by western blotting. In addition, western blots were run on homogenates of ERA- and ERB-transfected cells. For example, antibody from clone 6A12 was specifically bound in the ERB-transfected cells and did not bind to the ERA-transfected cells.

Example 4: Immunocytochemical Analysis

Both ERA- and ERB- transfected cells were reacted with antibody from clone 6A12 and the binding of the antibody visualized using immunoperoxidase staining techniques. The 6A12 antibody had no cross-reactivity or staining in the ERA-transfected cells, while the control ERA antibody showed nuclear staining. In contrast, in ERB-transfected cells, 6A12 antibody showed both nuclear and cytoplasmic staining while the control ERA antibody showed no cross-reactivity or staining.

Antibodies from the ERB clones were also tested to verify that they would stain fixed, parrafin-embedded tissue samples. Six samples of breast tumors, 3 tumors being ERA-positive and 3 tumors being ERA-negative, were stained with the anti-ERB antibodies. All six tumor samples stained with the anti-ERB antibody verifying that these antibodies stained archival tumor samples.

Slides were made of tumor sections cut 3-4 microns thick and baked overnight at 58° C and deparaffinized in a Shandon-Lipshaw Varistain™, Shandon-Lipshaw Manufacturing Corporation, Pittsburgh, PA. Slides were removed from the Varistain and transferred to a container containing 1X Tris-buffered saline.

For heat-induced antigen retrieval, all antibodies were run using 0.1 M Tris-HCl (pH 9.0). Antigen retrieval was achieved by placing the slides in an infrared hot plate/ pressure cooker at 120°C for 5 minutes. Slides were rinsed in nanopure water (six changes) and placed in 1X Tris-buffered saline. Endogenous peroxidase was blocked using a 3% hydrogen peroxide solution for 5 minutes at room temperature and then rinsing with nanopure water (three changes). Blocked tissue sections were placed in 1X Tris-buffered saline for at least 5 minutes.

The Vector avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) was used to block non-specific binding; this involved placing 3 drops of avidin solution A into each well formed by the slide-coverplate complex and incubating for 15 minutes, rinsing each well with 1X Tris-buffered saline, draining each well, placing 3 drops of biotin B solution into each well and incubating for 15 minutes, rinsing each well as before, and finally draining each well. Secondary antibody was then applied (biotinylated rabbit anti-mouse; 1:200 dilution; Dako, Carpinteria, CA) and incubated with the sections for 30 minutes. Sections were rinsed with 10X Tris-buffered saline before the application of a freshly prepared horseradish peroxidase labeled strepavidin (Dako, Carpinteria, CA) at a 1:200 dilution. The horseradish peroxidase labeled strepavidin was incubated with the samples for 30 minutes at room temperature. The slides were then stained with diaminobenzidine by incubating each section for 15 minutes at room temperature with three drops of diaminobenzidine solution and then rinsing each section in six changes of nanopure water, followed by transfer of the sections to a humidity chamber. After wiping the excess nanopure water from each slide, three drops of 0.2% osmium tetroxide were applied to each slide and the slides incubated for 30 seconds at room temperature. The sections were rinsed in six changes of nanopure water. All slides were counter-

stained with 0.05% methyl green for 30 seconds. The stained slides were dehydrated, cleared and mounted.

Figure 5 is an example of an immunocytochemical analysis of paraffin-embedded fixed tissues processed with 14C8 ER-β monoclonal antibody. In Fig. 5A, endocervical epithelium tissue is treated is shown. Fig 5B, illustrates similarly treated tissue from infiltrating lobular carcinoma of the breast.

Example 5: Immunocytochemical Diagnostic Kit

One embodiment of the present invention is a kit for detection of ERB in biological samples. Such a kit includes, but not limited to, a kit for immunocytochemical analysis of tissue samples. Such immunocytochemical kits are routinely used in the diagnosis and monitoring of diseases such as cancer as these techniques allow one of skill to recognize normal versus abnormal structures in individual cells and to link those abnormalities to some marker of disease, such as the presence of ERB.

The kit of the present invention would include ready-to-use monoclonal antibodies of the present invention that target the ERB. In a preferred embodiment, the kit would contain the monoclonal antibody 14C8. A kit of the present invention would include a blocking reagent of normal rabbit serum, a primary antibody (14C8), a biotinylated rabbit anti-mouse secondary antibody, avidin, biotinylated horseradish peroxidase, hydrogen peroxide solution, and diaminobenzidine tablets.

Example 6: Recognition of All ER-Beta Forms in Tissues

Antibodies that recognize all forms of ER-β were characterized in a manner similar to those for the forms discussed above. These antibodies were used to develop and characterize antibody reagents generated specifically against estrogen receptor beta isoforms ER-β2-5 and ER-βcx. Alterations in the amounts and/or cellular localization of ER-β and its isoforms is likely a clinically important biological feature in tumors where ER-β is expressed (e.g. breast, ovary, prostate). The analysis of ER-β and determination of the protein levels for the specific isoforms will assist the clinician to detect, treat and prevent breast, as well as other cancers (e.g. ovarian, prostate). Testing will be accomplished by:

1. Prepare the molecular reagents necessary for analysis of estrogen receptor beta (ER-β) in breast and other cancers. cDNA probes and gene specific primers using nonconserved nucleotide sequences will be used to analyze mRNA levels. Polyclonal and monoclonal antibodies will be produced that specifically recognize each isoform of ER-β. These will be used for the detection of protein levels in direct immunoblotting, immunoprecipitation, immunofluorescence and immunohistrochemistry experiments.

2. Determine and correlate the protein and messenger RNA expression levels, in normal and tumor cell lines.

3. Measure levels of the ER-beta isoforms in 200 human breast tumor samples from the Baylor SPORE Breast Cancer Developmental Bank. Determine how the levels and/or

- cellular localization (cytoplasmic vs. nuclear) correlate with other prognostic indicators, including ER, PgR, DNA ploidy, S-phase fraction, p53, HER-2/*neu*, or histologic grade.

Optimal conditions will be determined using standard approaches know well to those of skill in the art of immunohistochemistry that will give adequate and specific immunostaining in frozen and archived formalin-fixed, paraffin-embedded tissue/tumor sections isolated and processed over a wide range of conditions. Tumor analysis will be extended by doing a large retrospective study of patients that received no systemic adjuvant therapy following local surgery. This type of study will provide an ideal setting for evaluating the importance of ER-β and ERB isoforms as potential prognostic factor in breast cancer.

Preliminary Data.

To generate the ER-β monoclonals a fusion protein containing amino acids 1-153 was used which has very little homology to ER-α. The antibodies tested have been mapped to two regions of the ER-β protein. For the monoclonal antibodies 14C8 and 4D2 the epitope is located within the first 36 amino acids and for clones 6A12 and 6B12 the epitopes are located within amino acids 36-153.

Results of immunoblotting using *in vitro* translated protein indicate that none of the antibodies cross reacted with ER-α and confirm the epitope mapping results. A summary of the initial immunohistochemistry results using breast cancer tissue are shown below. These results indicate that one of the antibodies gives a strong specific nuclear signal in a ER-β positive tumor

indicating that this antibody is recognizing ER-β in formalin-fixed paraffin-embedded tissue and will be a useful reagent.

	14C8	6B12	6A12
WB with IVT protein	+, single band, FL	+, 2 bands, FL and a smaller band	+, 2 bands, FL and a smaller band
Permanent IHC	Good nuclear signal	Weak cytoplasmic signal	N/A
Conclusion	Best of any reagent at this point.	No further testing	

In a comparison to other antibodies available, none gave nuclear staining in our immunohistochemical assays using formalin-fixed paraffin-embedded tissues. These include the antibodies used by Fuqua, et al. (Fuqua, S.A.W. et al. 1999. *Cancer Res.* 59:4525-4528) analyzing ER-β protein expression in breast cancer cell lines and tumors. While these antibodies worked well in immunoblotting and immunoprecipitations, the polyclonal antibody and monoclonal antibody both gave weak cytoplasmic staining. Commercially available antibody used in the study by Jarviene, et al., (Jarvinen, T.A.H. et al. 2000. *Am J Pathology* 156:29-35) (PA1313 from Affinity Bioreagents, Inc.) did not work on paraffin-embedded tissue and only gave a weak cytoplasmic stain. These results confirm previously published results and indicate the importance of generating useful reagents to between detect ER-β in clinical samples.

EXPERIMENTAL DESIGN AND METHODS FOR EXTENDING USEFULNESS OF REAGENTS, TESTS AND KITS OF INVENTION

To prepare the molecular reagents necessary for analysis of estrogen receptor beta in breast and other cancers, the following steps will be taken. cDNA probes and gene specific primers using nonconserved nucleotide sequences will be used to analyze mRNA levels. Specific polyclonal and monoclonal antibodies will be produced that specifically recognize each isoform of ER-βeta. These antibodies will be tested for single band specificity by direct western blots and used for the detection of protein levels in direct immunoblotting, immunoprecipitation/western blotting, immunofluorescence and immunohistochemistry experiments in normal and tumor cell lines and tissues.

In order to understand the role of estrogen receptor beta (ER-β), it will be necessary to first obtain the molecular reagents necessary to analyze ER-beta's expression and dissect ER-beta's mechanism of action. These reagents will allow determinations for each ER-β isoform if there is a direct link between alterations in mRNA levels, protein expression and possibly cellular localization in normal and cancer cell lines and tissue samples. Therefore, isoform specific DNA primers will be utilized and will be used to perform RT-PCR that will allow measurement of isoform specific mRNA levels. To measure protein levels, it will be necessary to generate a series of polyclonal and monoclonal antibodies that are specific for each ER-β isoform and give single-band specificity in a direct western blot. These reagents can then be tested and developed to provide information that can be used to profile malignant cells so that clinicians can

recommend the most effective treatment regimens for their patients.

Experimental Methods.

Isolation of molecular probes for recombinant protein production and expression.

The isolation of a cDNA for each ER-β isoform will be accomplished by PCR based strategies using synthetic oligonucleotides. Poly A+ RNA will be isolated from human cell lines that have been shown to express the ER-β isoforms and RT-PCR will be performed (Asubel, F.M. et al. 1998. *J. Wiley and Sons*). To generate the complete coding region for each protein, it will be necessary to use the previously published sequences for each isoform, and specific synthetic oligonucleotides will be synthesized corresponding to the initiating ATG and 3' of the termination codon of the protein (Moore, J.T. et al., 1998. *Biochem BioPhys Res. Comm.* 247:75-78; Fuqua, S.A.W. et al. 1999. *Cancer Res.* 59:4525-4528). Artificial restriction sites will be added to each oligonucleotide to allow directional in-frame cloning into a vector to allow cRNA synthesis as well as expression in prokaryotic and eukaryotic cells.

Cloning and purification prokaryotic recombinant proteins.

The PCR-generated coding regions for the ER-β isoforms will be fused in-frame into the appropriate vector pET28(a-c) (Novagen, Madison, WI). This vector system uses the T7/lac promoter (Studier, F.W. et al. 1990. *Methods Enz* 185:60-89) that contains the *lac* operator sequence for more stringent suppression of basal expression. It also contains an amino terminal

and carboxyl terminal histidine peptide sequence tag (6His-Tag) for affinity purification of the recombinant protein. The recombinant protein can be identified using a His-tag antibody. This vector also contains the T7 promoter which will be helpful in accomplishing our other specific aims since it allows *in vitro* synthesis of our cDNA which can be translated and labeled for *in vitro* binding studies (see Figure 3). These cDNAs cloned into this vector will also be used to generate site directed point mutants that change the putative phosphorylated amino acids for each of these proteins.

Initially, the *E. coli* BL21 or BL21LysS bacterial strains (Studier, F.W. et al. 1990. *Methods Enz* 185:60-89; Smith, D.B. et al. 1988. *Gene* 67:31-40) will be used as well as the standard induction and isolation procedures that have been established and used successfully in the laboratory. After the induction of the recombinant fusion protein (with IPTG), examination will be made of the whole cell lysate by SDS-PAGE. If the fusion protein is expressed well, a determination will be made to see if it remains soluble or forms insoluble inclusion bodies within the bacteria. If the recombinant protein is soluble, it will be purified using nickel-agarose beads (Smith, D.B. et al. 1988. *Gene* 67:37-40). The recombinant protein will be eluted with imodizole, and the integrity of the protein determined by staining SDS-PAGE gels with Coomassie Brilliant Blue. The amount of protein will be quantitated by the method of Bradford (Bradford, M.M. et al. 1976. *Anal biochem* 72:248-254). If the protein is largely insoluble, it may be necessary to modify our induction protocol by changing temperature before/after induction, time of cell harvest after induction and IPTG concentration used for fusion protein

induction. If the protein remains completely insoluble after these modifications, the protein will be denatured using urea or guanidnium hydrochloride and slowly renatured by dialysis with decreasing concentrations of the denaturant to obtain soluble protein.

Although histidine tagged recombinant proteins have provided a simple method to obtain highly purified protein, problems in level of expression and solubility of the protein may occur. If this happens, it will be necessary to use a different expression system. Since expression using other bacterial systems (pGEX, GST-fusion, etc.) may have similar problems, it may become necessary to employ the baculovirus system to express these troublesome proteins. The cDNAs will be cloned into a baculovirus expression vector pMelBac (A, B and C)(Invitrogen, Carlsbad, CA) and co-transfected with Bac-N-Blue DNA, pure recombinant plaques will be isolated, then amplified and infected into "High Five" insect cells which provide higher levels of expression than Sf9 insect cells. Alternatively, a yeast system can be used, which has the advantage of yielding proteins with mammalian-cell-like post-translational modifications.

Cloning of eukaryotic expression vectors.

All ER-β isoform cDNAs will be cloned into the pcDNA3.1 (Clonetech, Palo Alto, CA) expression vector in order to analyze antibody specificity. These vectors will also be used in future studies on receptor-receptor interaction and the effects of specific estrogenic compounds and estrogen agonists and antagonists.

Generation and screening of monoclonal antibodies.

Generally, polyclonal and monoclonal antibodies will be produced using, fusion proteins and synthetic peptides to generate specific epitopes to distinguish the protein isoforms. For each recombinant protein/synthetic peptide antigen, 5 six week old Balb/C female mice will be obtained from Harlan Labs and housed at the UTHSCSA animal facility (LAR-UTHSCSA; San Antonio, Texas) will be injected. Blood from all animals that give a good immune response will be collected after the second and third boosts, the polyclonal serum pooled and used for preliminary experiments. Two animals with the highest titer of specific antibodies for monoclonal production will be utilized. If necessary a second fusion will be done using the other animals. Freund's adjuvant will be used because of its ability to stimulate a strong and prolonged immune response. The adverse side effect is the potential for invoking granuloma formation. To avoid this, injections will be made using complete Freund's adjuvant (CFA) in the primary injection and use will be made of incomplete Freund's adjuvant (IFA) for boosts. Serum will be collected to analyze the titer of serum antibodies, 5-7 days after each boost. Normally 100-200ul of blood and will not exceed more than 300ul. The mice will bled by nicking the vein behind the eye with a size 11 blade. The animals will be anesthetized using metafane, which will be administered before the procedure via inhalation. Using 5 animals per antigen will also allow obtaining sufficient amounts of polyclonal antibody from serum bleeds to initiate the research experiments while preparing monoclonal antibodies. All procedures will be done according to IACUC-approved protocols.

The injection protocol is listed in the table below.

Agent	Dose, Volume, Vehicle	Route of Administration	Frequency of Administration	Anticipated Effects
Antigen	50ug, 100ul., CFA	subcutaneous (3 sites)	1	
Antigen	50ug, 100ul, IFA	subcutaneous (3 sites)	2-3x @ 3 weeks	Immune Response
Antigen	50ug, 100ul, PBS	intravenous (tail vein)	1	granuloma formation

More specifically, monoclonal antibody production using synthetic peptides that are specific for each ER-β isoform will be performed. Groups of 5 mice will be immunized with one peptide. The initial injection will be using 150ug of purified protein or synthetic peptide that has been coupled to Keyhole Limpet Hemocyanin (KLH) or Multi-Antigenic Peptide (MAP) with complete Freunds adjuvant (CFA) per mouse subcutaneously. Two more immunizations will be performed at 2-3 week intervals, using similar amounts of antigen in incomplete Freunds adjuvant (IFA). After the second immunization and each subsequent injection, the titers for specific polyclonal antibodies will be tested by ELISA. To obtain polyclonal antibodies for initial experiments, the mice will be bled by nicking the vein behind the eye (100-200ul) one week after each boost. After the titer for the peptide-specific antibodies has been induced to appropriate levels, the best responding mouse for each protein will be used for monoclonal antibody production. The final immunization, without adjuvant, will be by tail vein injection. Three days later the mice will be sacrificed. Spleens will be harvested and prepared for PEG-mediated cell fusion with myeloma NS-1 cells (Zhu, X. et al. 1995.

J Biol Chem 270:19545-19550; Chen, Y.M. et al. 1995. *Science* 270:789-791 54). The fusions will be plated by limiting dilution, and the resulting hybridoma supernatants will be tested by ELISA for reactivity with the appropriate recombinant protein. The initial positive supernatants will then be propagated and retested by ELISA, with the resulting secondary-screen positive supernatants being used for immunoblotting (see below). These procedures have been used successfully by the inventors in the generation of other monoclonal antibodies such as those made against BRCA1, ER-Alpha, ER-βeta, PgR and SMRT.

After confirmation that each hybridoma is producing antibodies that show specificity by immunoblotting using the recombinant proteins produced above, they will be single-cell cloned to generate a pure population of antibody-producing cells. Identification will be made of about 10-15 positive hybridoma supernatants for each fusion that will be characterized.

The inventors have found that is critical to generate monoclonal antibodies for detection of estrogen receptor epitopes in tumor tissue samples. They have found and disclose for the frist time herein that while most antibodies will work well on sections that have been fixed with organic solvents or paraformaldehyde, sometimes the epitope is hidden by a cell structure that may be unmasked with protease treatment, or the epitope is destroyed during fixation. If this occurs with one of the antibodies to an isoform that has been determined to have altered expression levels/localization (which, therefore makes it a good candidate for diagnostic use), it will be necessary to generate new antibodies specifically for use in immunohistochemistry. One possible way to generate antibodies that will not be affected by the fixation procedure is to immunize mice

with paraformaldehyde-treated antigens and to screen resultant hybridomas by immunohistochemistry.

Correlation of Protein and Messenger RNA Expression Leveles inTumor/Normal Tissue

Recent evidence has shown that expression of ER-β and ER-β isoforms are expressed in normal breast epithelial cells and breast tumors and breast cancer cell lines. Using isoform specific synthetic oligonucleotide primers, measurements will be made of the mRNA levels by RT-PCR and proteins levels using the isoform specific antibodies generated above. These studies will extend the previously published work and analyze a number of different normal and cancer cell lines (breast, ovarian, prostate). Comparisons will be made of the levels of ER-β and ER-β isoforms proteins by direct western blot and/or immunoprecipitation (IP)/western blot analysis and the cellular localization by immunofluorescence and immunohistochemistry in these human cell lines.

Analysis of Estrogen Receptor-Beta protein in normal and cancer cell lines

It has been shown previously that the mRNA levels of ER-β isoforms is cell line dependent. Approximately 20 different breast cancer cell lines (e.g., MCF-7, T47D, BT-20) and normal breast epithelial cells (e.g., HBL-100), as well as several ovarian (e.g., CAOV3, SKOV3) and prostate (e.g., DUI45) cancer cell lines will be analyzed for their expression of each ER-β isoform at the protein and mRNA level. Briefly, to analyze protein levels, 1-5 x 10^6 cells will be harvested in either EBC buffer (50mM Tris pH7.4, 120mM NaCl, 1mM EDTA, 1mM β-mercaptoethanol, 0.5% NP-40) or lysis 250 buffer (50mM Tris pH 8.0, 250mM NaCl, 5mM EDTA, 50mM NaF, 0.1% NP-40). The

extracts will be incubated at 4 C for one hour and centrifuged, and the insoluble material discarded. The protein concentration will be determined by the Bradford method, and 100-200μg of lysate will be used. SDS/PAGE, direct western and immunoprecipitation/western blotting analysis will be performed using standard procedures. Only antibodies detecting a band of the appropriate size for the endogenous protein will be used for testing in immunofluorescence (IF) assays (see below).

Method to analyze mRNA in test cell lines

The levels of ER-β mRNA have been shown to be altered in breast cancer, and a determination will be made to if there is also a correlation of ER-β protein level with mRNA levels in the test cell lines. These experiments will also provide a secondary method of analysis for breast cancer specimens that cannot be analyzed by IP/western blotting or immunohistochemistry. Similar primers to that which have been previously used will be used here to determine ER-β isoform mRNA levels (Moore, J.T. et al. 1998. *Biochem BioPhys. Res. Comm.* 247:75-78; Fuqua, S.A.W. et al. 1999. *Cancer Res.* 59:4525-4528). Initially, semiquantitative PCR methods will be used to analyze the amounts of ER-β isoform mRNA in these cell lines. Briefly, total RNA will be extracted from cell monolayers, the concentration will be determined, and serial dilutions of each sample (50, 25, 12.5 6.25ng) will be added to the GeneAmp PCR kit (Perkin-Elmer) that allows reverse transcription and subsequent amplification of the synthesized cDNA. Specific primer for each ER-β isoform and b-actin (internal control) will be used. Samples will be analyzed by gel electrophoresis, fluorescence quantitated, and results standardized to b-actin.

Analysis of ER-β isoform expression and cellular localization in cancer cell lines

The normal breast epithelial cell lines and various cancer cell lines will also be evaluated for expression of ER-β protein isoforms using standard immunofluorescence and/or immunohistochemical (IHC) methodologies. For immunofluorescence cells will be seeded on glass cover slips and processed using fluorescent labeled secondary antibodies. For immunohistochemistry, concentrated pellets of each cell line will be processed to paraffin blocks, and histological sections from the blocks will be the substrated for the IHC assays. In these protocols, the previously produced ER-β monoclonal antibodies will be used, as well as any new isoform specific monoclonal antibodies that have been tested in immunoblotting of control and cancer cell lines. All protocols will use several dilutions to eliminate any nonspecific cross reactivity that has been previously reported as a potential problem in staining for ER-α. As positive controls for staining each cell line will also be transfected with the corresponding isoform expressing eukaryotic vector.

Immunofluorescence in cell lines

Since the potential exists that the monoclonal antibodies will work in a western and/or immunoprecipitation assay, but not detect the antigen in whole cells, it may be necessary to analyze cellular localization by immunofluorescence. Cells that have been fixed using organic solvents (methanol) or paraformaldehyde will be used to determine if the monoclonal antibodies will have cross-reactivity in cell staining procedures by indirect immunofluorescence using a flurochrome-

conjugated secondary antibody. The monoclonal antibodies will be tested using both hybridoma culture supernatants and purified antibodies isolated from the supernatants. The monoclonal antibody against an 84kDa nuclear matrix protein will be used as a positive control for nuclear staining. This protein, initially isolated by its interaction with the amino terminal region of the retinoblastoma protein, has been used extensively as a nuclear-specific marker in both immunofluorescence and cell fractionation studies (see Figure 2 ref. 71). Specifics of the basic staining procedure for immunofluorescence are as follows:

1. Plate $8x10^3$ cells/well in a 24 well dish on glass coverslips (+/- poly-lysine).
2. After 20-24 hours, rinse cells briefly with PBS.
3. Fix cells either with 4% paraformaldehyde in PBS or (a) 1% formalin, (b) methanol, (c) acetone.
4. Rinse with PBS.
5. Incubate with 0.1% NP-40 in PBS (or Triton-X100).
6. Wash 2x with PBS.
7. Incubate samples in 4-10% goat serum for 1 hour at room temperature.
8. Wash with 1% BSA in PBS.
9. Incubate primary antibody for 1 hour at room temperature or overnight at 4°C.

10. Wash 3x with 1% BSA in PBS.

11. Incubate with fluorescent secondary antibody for 45 minutes at room temperature (in dark).

12. Wash extensively (at least 3x) with 1% BSA in PBS.

13. Counterstain with DAPI to visualize nuclei if desired.

14. Mount coverslips with aqueous mounting medium or 90% glycerol in PBS.

15. Examine slide using fluorescence microscope, store slides at 4°C in dark.

This basic procedure will be used initially to test the sensitivity of the antibodies. Each antibody will have to be optimized by varying the primary antibody concentration and/or incubation time, and other parameters involving the cell preparation, fixation technique, primary and secondary antibody binding and the method of detection.

Immunohistochemistry using paraffin block preparation (tissue culture cells/tissue).

Each cell line for each experimental condition will be grown to high density in one T150 tissue culture flask. When the cells are ready to harvest, they will be gently scraped from the flask bottom using a sterile rubber spatula. The free-floating cells will be washed, pelleted, resuspended in 10% neutral buffered formalin for 6 hours fixation, pelleted again, and resuspended in 100 µl of 2.5% warm agar in a microfuge tube. The agar quickly hardens into a gelatinous pellet of cells that can be processed to a paraffin block just as if it were a routine tissue biopsy. Histological sections

will be cut from the blocks at 4 μm and float-mounted on adhesive (silanized) glass slides.

A standard, sensitive, generic detection system will be used with each ER-β primary antibody. This promotes both technical and cost efficiency and uniformity of results. Each antibody may require certain minor modifications for optimum performance, which usually involves adjustments of antibody concentration, incubation time, or the use of various antigen-enhancement techniques. The detection system is based on linking the primary antibodies to biotinylated secondary antibodies to peroxidase-conjugated streptavidin. The signal is developed with diaminobenzidine/hydrogen peroxide as the chromogen, which is enhanced with osmium tetroxide, and contrasted to alight methyl-green counterstain. Specifics are as follows:

Generic Detection System

1. Preparing sections

   - cut at 4μm
   - float-mount on PLUS-coated slides in an adhesive free water bath
   - dry overnight at room temperature

2. Deparaffinizing

   - graded xylene, alcohols, and distilled water

3. Antigen retrieval (depending on primary antibody)

   - none, or
   - 0.1 M citrate buffer at pH = 6.0 for 5 minutes in pressure cooker at boil, or
   - pronase E at 1 mg/ml in PBS for 2 minutes

4. Block endogenous peroxidase

   - 0.1% sodium azide/3% hydrogen peroxide in automation buffer for 15 minutes

5. Block non-specific protein (antibody) binding

   - 10% chicken ovalbumin in Tris saline for 10 minutes

6. Primary antibody

   - 2 hours to overnight (depending on antibody) at predetermined concentration in diluent buffer

7. Linking antibody (Dako, Carpinteria, CA)

   - biotinylated anti-mouse or anti-rabbit antibody at 1:100 in diluent buffer for 1 hour

8. Streptavidin-peroxidase conjugate (Dako, Carpinteria, CA)

   - 1:100 in diluent buffer for 1 hour

9. Chromogen

   - diaminobenzidine at 1 mg/ml in automation buffer with 1 µl H_2O_2 for 30 seconds to 10 minutes

10. Signal intensification

    - 0.2% osmium tetraoxide in PBS for 1 to 10 minutes

11. Counterstain

    - 1% freshly prepared methyl green for 5 minutes

12. Dehydration and mounting

    - graded alcohols and xylene

13. Coverslip

    - cover section with mounting media, Permount (electron Microscopy Sciences, Ft. Washington, PA) and glass coverslip

For details of procedures, see Durfee, T. et al. 1994. *J. Cell Biology* 127:609-622; Allred, D.C. et al. 1993. *J. Natl Cancer Inst* 85:200-206; Oesterreich, S. et al. 1996. *Clin Cancer Res* 2:1199-1206; Diab, S.G. et al. 1997. *Breast Cancer Res Treat* 43:99-103; Makris, A. et al. 1997. *Breast Cancer Res Treat* 44:65-74; Berardo, M. et al. 1998. *Cancer* 82:1296-1302; Allred, D.C. et al. 1993. *J Histotechnol* 16:117-120.

Expected Results:

From these experiments, we will complete out initial analysis of ER-β monoclonal antibodies. Using western blot analysis, we will eliminate antibodies that have nonspecific cross reactivity with other cellular proteins isoforms and ER-α and would give anomalous results for cellular localization and in immunohistochemistry. The preliminary analysis using immunofluorescence and immunohistochemistry using tissue culture cell lines allows the determination to be made of the potential usefulness of this technique for analysis of tumor samples and the sensitivity and specificity of each antibody.

Measuring ER-beta isoforms in archived breast tumor samples

To measure levels of the ER-beta isoforms in 200 human breast tumor samples from the Baylor SPORE Breast Cancer Developmental Bank, the following steps will be taken. It will be necessary to determine how the levels and/or cellular localization (cytoplasmic vs. nuclear) correlate with other prognostic indicators, including ER, PgR, DNA ploidy, S-phase fraction, p53, HER-2/*neu*,

or histologic grade. Currently, there are several types of markers that are used to make management and treatment decisions for patients with primary breast cancer. Prognostic factors are used to estimate the chances of disease recurrence if no systemic adjuvant therapy is given. Examples of prognostic factors are axillary lymph node status, tumor size, histologic grade, and steroid receptor status. Patients with a high estimated chance of disease recurrence more often receive systemic adjuvant therapy following local surgery. Predictive factors are used to select optimal therapies for individual patients.

Examples of predictive factors are steroid receptors, S-phase by DNA flow cytometry, and, more recently, HER-2/*neu* status (Allred, D.C. et al. 1998. *Modern Pathology* 11(2):155-168). Other newer markers such as heat shock proteins Oesterreich, S. et al. 1996. *Clin Cancer Res* 2:1199-1206; Elledge, R.M. et al. 1994. *Cancer Research* 54:3752-3757), p53 (Allred, D.C. et al. 1998. *Modern Pathology* 11(2):155-168), and apoptosis (Diaz-Cano, S.J. et al. 1997. *Diagn. Mol. Path.* 6:199-208) are not yet used routinely in the clinic, but they have provided important information about the biology of breast cancer, and their prognostic and predictive abilities are currently being evaluated.

ER-beta protein isoforms, which are involved in estrogen signal transduction and transcriptional regulation, may also be important prognostic and/or predictive markers for breast cancer and other types of cancer. To address this issue definitively, it will be necessary to correlate the level of these proteins with the clinical outcomes of patients with breast cancer. Required first steps before such definitive studies can be designed and conducted are to obtain preliminary information about the levels of these proteins that are present in human breast tumors, to determine

the variability of expression of these factors among tumors, to determine how often these factors are present in the cytoplasm or the nucleus, and to determine if the levels or the localization correlate other established prognostic or predictive factors. Since no correlation has been reported between expression and tumor grade and alteration in cellular localization, differential expression of the levels and localization among tumors may be seen. Associations with other prognostic factors that are known to correlate with tumor grade (e.g., steroid receptor status and S-phase fraction) may also be seen. The magnitude and variability of the expression of the ER-β isoforms and the correlation with other factors will greatly influence sample size requirements for subsequent correlative studies.

To address these issues, IHC will be used to test the antibodies of the invention for determining the levels and cellular localization in tumor samples from the Baylor SPORE Breast Cancer Development Bank. Many other factors, including ER, PgR, DNA ploidy, S-phase faction, p53, HER-2/*neu*, and histogolic grade have already been measured in these tumors, but no clinical follow-up is available for any of these patients. Results of these studies will be used to determine which proteins will be best for further study. These results will also be used to refine the sample size requirements for the follow-up study.

Methods.

Immunohistochemistry and Analysis of Protein Levels.

The levels of ER-βeta isoform specific antibodies will be determined by IHC using procedures previously described. ER levels and PgR levels have been previously determined by

biochemical assays (Dressler, L.G. et al. 1988. *Cancer* 61:420-427) and by IHC using the 6F11 antibody from Novocastra for ER (Harvey, J.M. et al. *J Clin Oncol* (in press)) and the KD68 antibody from Abbott Laboratory for PgR (6156); DNA ploidy and S-phase fraction have been previously determined by flow cytometry. (Dressler, L.G. et al. 1988. *Cancer* 61:420-427, Wenger, C.R. et al. 1993. *Breast Cancer Res Treat* 28:9-20); p53 has been measured by IHC using a cocktail of antibodies that includes Pab 1801 and Pab 240 from GeneTex (Allred, D.C. et al. 1993. *J Natl Cancer Inst* 85:200-206), and HER-2/*neu* has been previously measured by IHC using the TAB 250 antibody from Triton (Elledge, R.M. et al. 1998. *Clin Cancer Res* 4:7-12). Immunohistochemistry procedures will be as previously described.

In addition to the information and expression levels detected using IHC, the levels of protein will be determined by western blotting an/or immunoprecipitation/western blotting techniques. In these procedures pulverized samples of the identical tumor that was used in IHC will be lysed in 5% SDS, and the protein concentration will be quantitated using BCA reagent assay (Pierce, Rockford, IL). These levels can then be correlated to the scoring of the IHC and to the expression of the other predictive markers that have previously been documented from these tumor samples.

Statistical analyses

Each protein's levels will first be analyzed as continuous variables. For prognostic factors that are measured on a continuous scale (ER, PgR, S-phase fraction, p53, HER-2/*neu*), associations with each protein will be assessed by Spearman non-parametric correlation coefficients. For factors

that are inherently dichotomous or are usually classified into two categories (DNA ploidy, ER status, PgR status, p53 status, HER-2/*neu* status), Wilcoxon non-parametric rank sum tests will be used to compare protein levels. The distributions of IHC scores for each protein will then be examined to see if there any natural breakpoints that might be used to differentiate low levels from high levels, and appropriate procedures for binary variables (Chi-square tests, Fisher exact tests) will be applied. With a sample of 200 specimens, the width of a 95% confidence interval for rate of over-expression of each protein will be less than 14%. If the data follow normal distributions or can be transformed to normal distributions, an 80% power to detect a correlation of 0.195 and 90% to detect a correlation of 0.224 with any of the standard prognostic factors is predicted.